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TITLE: Novel Functions of EZH2 in Triple Negative Breast Cancer: Translation in to New Biomarker and Treatment Strategies.

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14. ABSTRACT In our third year of funding we have developed an antibody that recognizes the cytoplasmic phosphorylated EZH2 protein (pEZH2(T367)), and validated the specificity in human tissue samples by immunohistochemistry. Using this novel antibody, we found that pEZH2 (T367) expression in the cytoplasm increases with breast cancer progression, and that it is significantly inversely associated with H3K27me3 in breast cancer tissue samples. We have completed our binding assays using EZH2 and p38 proteins by quantitative bio-layer interferometry (BLI) and conclusively demonstrated a strong binding affinity of EZH2 for p38 α (binding affinity KD of 5.54 nM). We successfully optimized a proximity ligation assay (PLA) to investigate the binding of these proteins in situ. This assay confirmed that EZH2 binds to p38 in breast cancer cell lines and that the binding occurs predominantly in the nucleus of breast cancer cells, which was previously unknown. We have developed various EZH2 mutants that will be helpful in identifying the required EZH2-p38 binding sites.					
15. SUBJECT TERMS Triple negative breast cancer, EZH2, disparities					
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1. INTRODUCTION

This is the third annual report for a project that aims to understand the non-canonical functions of EZH2 as a determinant of breast cancer invasion and metastasis, and to elucidate the relevance of cytoplasmic expression of EZH2. EZH2 (Enhancer of Zeste Homolog 2), a Polycomb group protein presumed to function in controlling the transcriptional memory of a cell [1], is up-regulated during progression from ductal carcinoma in situ, the precursor of invasive carcinoma, to invasive carcinoma and distant metastasis [2]. Furthermore, EZH2 protein is over-expressed in 55% of invasive breast carcinomas, and is significantly associated with poorly differentiated tumors [2-4]. Our laboratory has previously shown that EZH2 is a powerful independent prognostic biomarker in breast cancer, providing outcome information above and beyond conventional prognosticators used in the clinical setting [2]. We have also demonstrated by Kaplan-Meier analysis that tumors with high EZH2 expression had a worse disease free and overall survival than tumors with low EZH2 expression. Our recent studies support that EZH2 is expressed in the cytoplasm in aggressive breast carcinomas from Ghanaian women [5]. Furthermore, we have found that EZH2 binds to p38 and AKT1 in breast cancer cells, leading to their methylation. These data led us to hypothesize that EZH2 is expressed in the cytoplasm of a subset of TNBC tumors where it methylates and activates p38 and AKT1 leading to metastasis. We further hypothesize that detection of cytoplasmic EZH2, phosphorylated p38 and/or phosphorylated AKT1 proteins may identify TNBC tumors with more aggressive behavior and heightened metastasis. As I will illustrate below, we have made progress in this second year of funding.

2. KEYWORDS: triple negative breast cancer, EZH2, African, health disparities.

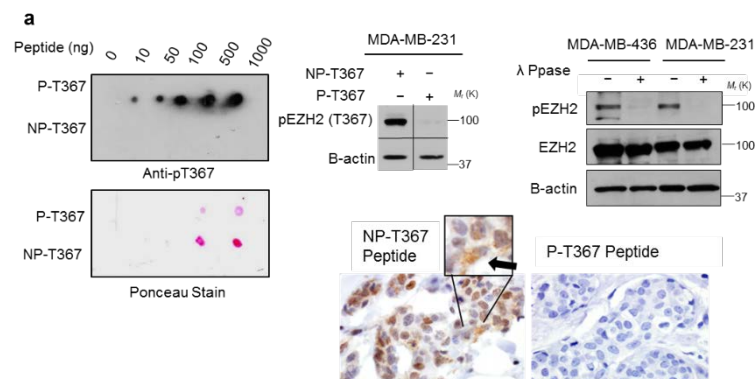
3. ACCOMPLISHMENTS

Below are brief descriptions of key accomplishments according to the approved statement of work for Year 1.

Aim 1: To investigate whether the concordant expression of cytoplasmic EZH2, phospho-p38 and/or phospho-AKT1 in tissue samples identifies TNBC with high metastatic ability in African, AA, and White women.

- **Task 1:** To test the pattern of expression of EZH2, phosphorylated p38, phosphorylated AKT1, and H3K27me3 proteins in invasive carcinomas of women from different races (Years 1-1.5).
- **Task 2:** To investigate associations between expression of cytoplasmic EZH2, phospho-p38, phospho-AKT1, and low H3K27me3 with race, TNBC phenotype, clinical and pathological features, and patient survival (Years 2-3).

During this year we have successfully performed the tasks proposed. Our lab has developed an antibody that recognizes the site of p38-mediated phosphorylation on EZH2 protein (pEZH2(T367)). We



validated the specificity of this antibody for western blot and immunohistochemistry in breast tissue samples (**Fig. 1**). Using this antibody, we found that pEZH2 is expressed in the cytoplasm of breast cancer cells and that its expression increases with breast cancer progression (**Fig. 2**).

Fig.1. pEZH2(T367) can be detected *in vitro* and *in vivo*. Related to Figure 1. a. Dot-blot assays were performed using increasing quantities of

phosphorylated (NSSRPS(pT)PTINVL) or non-phosphorylated (NSSRPSTPTINVL) peptides after incubation with affinity purified pEZH2 (T367) antibody. Ponceau stain shown below as loading control. **b.** Peptide competition western blot of MDA-MB-231 whole cell lysates using pEZH2 antibody pre-incubated with 200-fold molar excess of either non-phosphorylated or phosphorylated peptide. **c.** Western blot using MDA-MB-436 and MDA-MB-231 whole cell lysates treated with lambda phosphatase to dephosphorylate protein. **d.** Peptide competition immunohistochemistry of an invasive breast carcinoma using pEZH2 antibody pre-incubated with 200-fold molar excess of either non-phosphorylated or phosphorylated peptide.

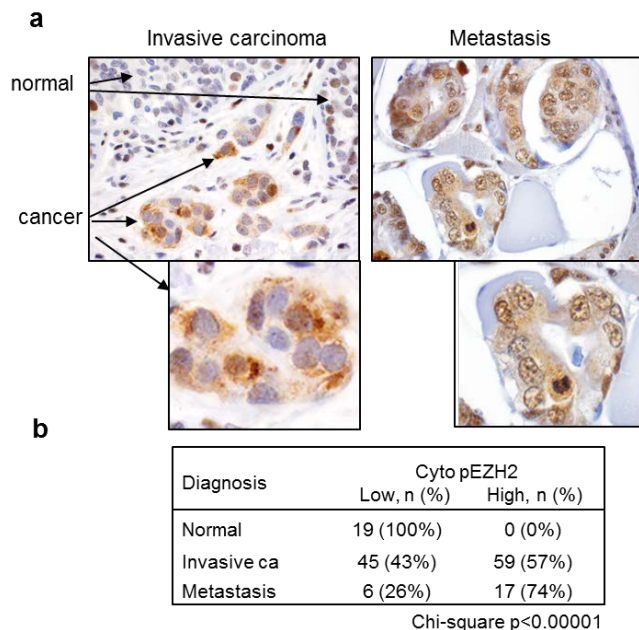


Fig.2. Phosphorylated EZH2 (T367) is expressed in the cytoplasm of invasive breast carcinoma and distant metastases. **a** Immunohistochemical analysis of pEZH2(T367) expression using a specific antibody in human tissue samples of 193 patients. Pictures show a representative invasive breast carcinoma with adjacent normal breast (left) and metastasis (right) (400x magnification). Insets show expression of pEZH2(T367) in cancer cells (600x magnification). **b** Results are tabulated. Cytoplasmic pEZH2 is significantly associated with invasive carcinoma and metastasis compared to normal and fibrocystic changes (Chi-square $p < 0.00001$).

We also, as planned, immunostained our human breast cancer TMAs with anti-H3K27me3, showing a significant inverse association between pEZH2 and H3K27me3. In year 3, we have completed out immunostaining studies of human breast cancer

samples and found that pEZH2 (T367) is significantly associated with high histological grade and triple negative breast cancer status. These data have been published recently in Nature Communications [6].

Aim 2: Biochemical and biophysical characterization of the EZH2 interaction with p38 and AKT1 proteins and mapping EZH2 binding site that interacts with p38 and AKT1.

- **Task 3:** Determine the biochemical features of the EZH2 binding to p38 and AKT1 (Year 1-1.5).

Our studies in 1 and 2 of funding convincingly demonstrated that EZH2 binds to p38 with high affinity than to AKT1, and have elucidated the biophysical and biochemical characterization of EZH2-p38 binding using Quantitative Bio-Layer Interferometry (BLI) data with recombinant proteins. Our studies showed that EZH2 has a strong affinity for p38 α with binding affinity KD of 5.54 nM and kinetic constants: association rate of $k_{on} = 4.68 \times 10^6$ [M⁻¹s⁻¹], and dissociation rate, $k_{off} = 2.59 \times 10^{-2}$ [s⁻¹]. Affinity constant obtained from the kinetic analysis was in excellent agreement with the steady state analysis, KD of 3.4 nM, confirming the direct and strong binding interaction between EZH2 for p38 α . These data have been just published [6].

- **Task 4:** To test the binding of EZH2 to p38 and AKT1 in breast cancer cells and in cancer cells freshly isolated from patients with TNBC (Year 1-1.5).

Our initial studies demonstrated that EZH2 and p38 and p-p38 interact by co-immunoprecipitation. Recently, we validated a proximity ligation assay (PLA), which tests the presence of two proteins in close proximity (less than 40 nm apart), which strongly suggests direct binding. Using PLA, we recently showed that EZH2 and p38 interact in MDA-MB-231 cells, and that this interaction takes place primarily in the

nucleus [6]. We recently demonstrated that p38-mediated phosphorylation of EZH2 at T367 induces EZH2 cytoplasmic localization (**Fig.3**), and promotes invasion and metastasis of triple negative breast cancer cells [6]. These data open the way to further experiments to test function of pEZH2 (T367) in the cytoplasm and how this contributes to breast cancer metastasis, which is the subject of current investigations in our lab, which will be performed in the next year.

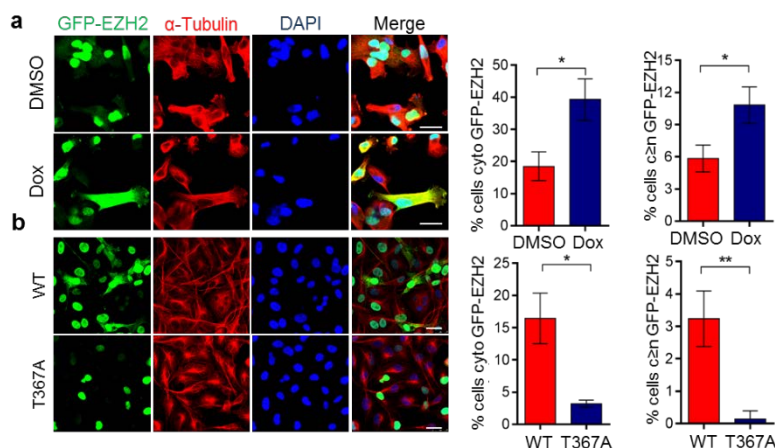


Fig. 3. p38-mediated phosphorylation at T367 promotes EZH2 cytoplasmic localization. **a** Immunofluorescence images of MDA-MB-231 cells transduced with lentiviruses to express GFP-EZH2 and a dox-inducible, constitutively active MKK6 kinase (MKK6EE). The percentage of non-mitotic cells expressing cytoplasmic EZH2 and cytoplasmic GFP-EZH2 \geq nuclear expression was quantified for >50 cells from three fields. Scale bars: 25 μ m. **b** Immunofluorescence images of MDA-MB-231 cells transduced with lentivirus to express GFP-EZH2 wild-type or T367A protein. The percentage of non-

mitotic cells expressing cytoplasmic EZH2 was quantified for >50 cells from three fields. Statistical analyses were performed using student's T-test. Scale bars: 25 μ m. Data for a-b shown as mean \pm SD and are representative from an independent experiment that was repeated with three biological replicates, each with at least three technical replicates. Statistical analyses were performed using student's T-test.

Aim 3: To elucidate the relevance of EZH2-mediated binding on TNBC invasion and metastasis *in vivo* and *in vitro*.

- **Task 5:** Investigate the importance of EZH2 binding to p38 and AKT1 in TNBC cell neoplastic functions *in vitro*. (Years 2-3).
- **Task 6:** Investigate the importance of EZH2 binding to p38 and AKT1 in TNBC growth and metastasis *in vivo*. (Year 3). These experiments will be carried out in the next cycle.

During this year, we made significant progress towards these tasks. By developing various EZH2 mutants, including a T367 phosphorylation deficient mutant (phospho-off), we have shown that EZH2 phosphorylation at T367 is required for invasion and metastasis [6].

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6. Anwar, T., et al., *p38-mediated phosphorylation at T367 induces EZH2 cytoplasmic localization to promote breast cancer metastasis*. Nat Commun, 2018. **9**(1): p. 2801.

Thus, the key research accomplishments in this year of work are:

- Characterized the expression of pEZH2(T367) in over 100 invasive carcinomas of the breast arranged in TMAs and demonstrated that it is associated significantly with triple negative phenotype and high histological grade.
- Detected pEZH2 (T367) in the cytoplasm of human breast cancers discovered that phosphorylation by p38 is required for EZH2 localization to the cytoplasm.
- Found that pEZH2 (T367) expression in the cytoplasm increases with breast cancer migration, invasion, and metastasis, and it is required for these events.
- Validated a specific binding of EZH2 and p38 proteins by Quantitative Bio-Layer Interferometry (BLI).
- Discovered that pEZH2 (T367) binds to cytoplasmic proteins, including the focal adhesion regulator Vinculin, which will be investigated in future studies.

We plan to continue our project as approved by the DOD.

This project has given me the opportunity to train several undergraduate and graduate students in the laboratory. Of note, this work was part of the graduate school thesis of Talha Anwar, who is the first author in our Nature Communications study. I gained from this experience by having time to teach and discuss important aspects of breast cancer histology and health disparities to my lab member during this year. We have also attended the AACR meeting in 2018 and presented the work. Attending the AACR meeting was extremely beneficial as we participated in minisymposiums, plenary lectures, and poster presentations which broadened our knowledge and understanding of breast cancer development and progression. We disseminated our results through poster presentations at AACR and University of Michigan meetings.

4. IMPACT

The main impact that our project has so far during these three years is the demonstration that EZH2 is expressed in the cytoplasm of TNBC and that it interacts strongly with MAPK p38 protein. We discovered that a consequence of EZH2-p38 binding is that p38 phosphorylates EZH2 at T367 which enables migration and invasion through binding to novel partners in the cytoplasm of breast cancer cells, which are under investigation. This is a **novel idea that has not been studied**, and, together with our future studies will represent a major paradigm shift in our understanding of how EZH2 drives TNBC, and may provide new biomarkers of aggressive breast cancer, and targeted therapies.

5. CHANGES/PROBLEMS

No problems to report. No changes to the original aims and tasks.

6. PRODUCTS

Anwar, T., et al., *p38-mediated phosphorylation at T367 induces EZH2 cytoplasmic localization to promote breast cancer metastasis*. Nat Commun, 2018. **9**(1): p. 2801.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

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Principal investigator (initiating PI)

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Contribution to project: planned direction of project to follow SOW, designed experiments and guided the student, analyzed tissue samples, oversaw construction of TMAs, analyzed results, assisted with writing of abstracts and posters.

Zaneta Nikolovska-Coleska, Ph.D.

Partnering PI

Nearest person month worked: 1

Contribution to project: planned direction of project to follow SOW, designed experiments and guided the postdoctoral fellow, analyzed and summarized the results.

Talha Anwar, B.S.

Graduate Student

Nearest person month worked; 6

Contribution to project: read the literature, designed experiments and discussed with PI, carried out experiments and analyzed the results, wrote abstracts and posters.

Lei Miao, Ph.D.

Postdoctoral Fellow

Nearest person month worked: 3

Contribution to project: read the literature, designed experiments and discussed with PI, carried out experiments and analyzed results.

8. SPECIAL REPORTING REQUIREMENTS

As this is a collaborative award, we are providing one report for both the initiating and partnering PIs, as requested by DOD.

APPENDIX, not necessary.